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Research Article

Fetal Exposure to Anti-Hypersensitive Drug, Zestoretic Delays Estrus Cycle and Suppresses Female Reproductive Potential in Rats at Their Adulthood

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Abstract

The current investigation is designed to explore the possible role of fetal exposure to the anti-hypersensitive drug zestoretic, a version of lisinopril (angiotensin-converting enzyme inhibitor) and hydrochlorothiazide (a diuretic drug) on female fertility efficacy in rats at their adulthood.The pregnant rats were oral gavaged withzestoretic 25, 50 and 100 mg/kg body weight on 7, 9, 11 and 13 days of gestation. The F1 female rats were tested with estrus cycle length and reproductive efficacy.Zestoreticfetal exposure induced significant change ($P<0.001$) in prolonged estrus cycles, reduced body weight, increased implantation loss and resorptions, conception time, mating index, and fertility index in female F1 rats, indicating potential harm to embryonic development and reduced fertility later in life.Though zestoretic reduces high blood pressure, its prenatal exposure harms F1 female reproductive health, causing prolonged estrus cycles, reduced fertility with increased conception time, resorptions and implantation loss. Although rat study results can't directly apply to humans, caution and strict dosage adherence are advised when using anti-hypersensitive drugs during pregnancy.

Keywords: anti-hypersensitive drugs, estrus cycle, fertility, female rats, reproduction and zestoretic

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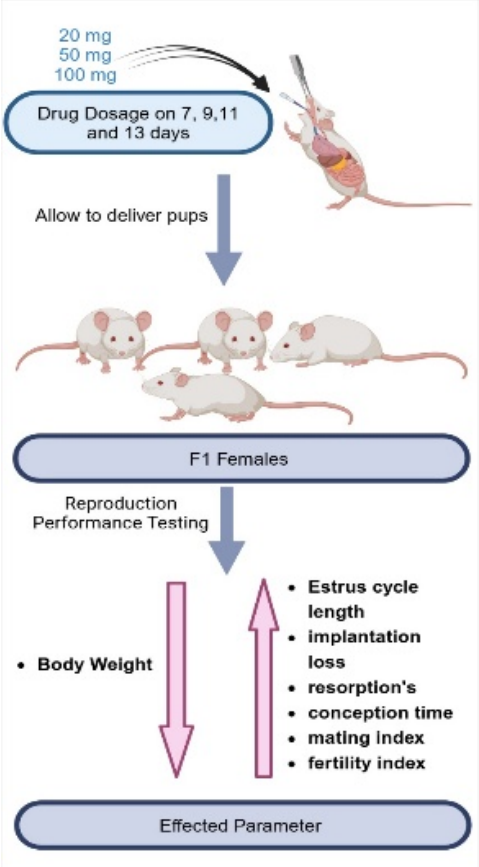

1. Introduction

Hypersensitivity is one of the global problems, as the consequences may lead to high blood pressure, heart attacks, and even, based on the severity; it leads to death [1]. To control hypertension, several anti-hypersensitive drug either individual or in combination of two drugs are available in the market. Zestoretic (ZES) is one of the anti-hypersensitive and diuretic drugs widely used to control hypersensitivity [2]. It is a mixture of two drugs, lisinopril (LIS) and hydrochlorothiazide (HCTZ) wherein LIS targets the renin-angiotensin-aldosterone system [3], while HCTZ targets renal tubular electrolyte (sodium) reabsorption. LIS is an inhibitor of angiotension converting enzyme (ACE) which mediates the synthesis of angiotensin II from angiotensin I [4]. This step leads to decreased vasopressor activity and reduced aldosterone secretion from the adrenal cortex. Finally, these events cause an increase in serum potassium concentration. On the other hand, HCTZ inhibits the sodium chloride cotransporter thereby prevent sodium resorption at the renal tubules and promote normal diuresis and natriuresis. Interestingly, as per the product monograph of LIS by [5], it has been shown that LIS may cause adverse effects like hepatotoxicity, hyperkalemia, reduced hematocrit and hemoglobin and proteinuria. The side effects of HCTZ appears to be dizziness, allergic reactions, vision related problems and also diabetogenic potential via aggravation of metabolic dysfunction. Moreover, hypersensitive patients require anti-hypersensitive drugs over long-term duration

and cannot be withdrawn suddenly. Despite of promising potential of these drugs, adverse side effects caused by these drugs questions the safety of these drugs and hence, the toxicity profile of these drugs needs to be evaluated.

Female reproductive system is controlled and coordinated by several factors including renin-angiotensin system (RAS). RAS regulation of oocyte maturation, thickening of endometrium lining, and hormone production has been demonstrated [6,7]. Prenatal period is one of the crucial periods during which the development of fetus occurs in terms of proper structural and functional integrity of vital organs later in life. Surprisingly, our previous studies have shown that prenatal exposure of rats to ZES caused developmental toxic effects in male and female F1 generation pups[8]. Exposure to ZES resulted in delayed vaginal opening and testicular descent in female and male F1 pups, respectively as compared to controls suggesting ZES at least in part interfere with the endocrine dependent developmental events in rats[8].

Human ovaries express almost all components of renin-angiotensin system (RAS). Therefore, it is conceivable that consuming anti-hypersensitive drugs by females especially during pregnancy may interfere with ovarian-RAS system which eventually targets female reproductive health. If so, what would be the fertility outcome of female progeny later in life. To test this hypothesis, the present investigation was evaluated to establish the role of prenatal exposure to ZES on female reproductive health in F1 generation rats at their adulthood by considering the reproductive endpoints such as body weights, estrus cycle and fertility efficacy.

2. Materials and Methods

2.1. Test chemicals

The experimental chemical, Zestoretic (ZES) tablets, were procured in the local medical agency, Life Pharma, Kadapa, AP, India. ZES is a combination of lisoprinol (LIS: (S)-1-[N2-(1-carboxy-3-phenylpropyl)-L-lysyl]-L-proline dihydrate), an ACE inhibitor, and hydrochlorothiazide (HCTZ: 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1dioxide), a diuretic drug. The chemical structures of LIS and HCTZ were shown in Figure **1**. Sodium chloride and other analytical grade reagents were obtained from HiMedia LaboratoriesPvt. Ltd., India.

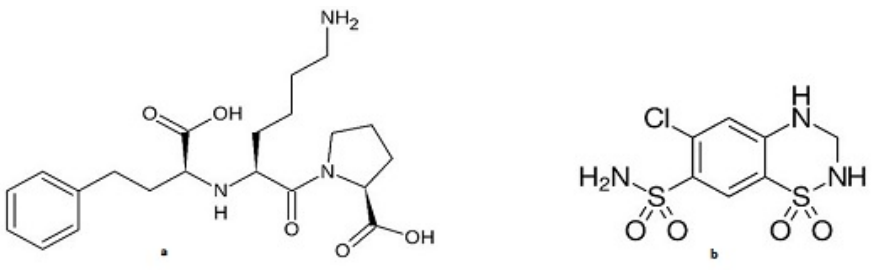

Figure 1: **a.** Structure of lisinopril; **b.** Hydrochlorothiazide.

2.2. Experimental design

Time-mated gravid female rats were randomly grouped into four (n =4/group) wherein rats in group I treated as controls and rats in groups II, III and IV were considered as experimental animals. The experimental design was as follows:

Group I: rats in this group received double distilled water (solvent vehicle used to dissolve ZES)

Group II: rats in this group orally gavaged with ZES at a dosage of 25mg/ Kg body weight

Group III: rats in this group orally gavaged with ZES at a dosage of 50mg/ Kg body weight

Group IV: this group rats are orally gavaged with ZES at a dosage of 100mg/ Kg body weight

The experimental chemical ZES is solvated in double distilled water and given to the time-mated gravid female rats on days 7, 9, 11 and 13 via gavage by adjusting the doses of ZES in accordance to the body weights of time-mated pregnant rats. All the rats belonging to the control and experimental groups were maintained on standard rat chow (S.V. Enterprises, Bengaluru and Karnataka, India) and supplied water *ad libitum*. Following completion of prenatal days, rats were allowed to give birth to young ones and weaned up to 21 days. The female rats were separated from all the groups and maintained in a demarcated cages up to 90 days for the determination of reproductive endpoints. The guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision on Animal Experiments, Government of India) and in compliance with Institutional Animal Ethical Committee at Yogi Vemana University, Kadapa, India (Resolution No: YVU/IEAC/PRR/13/2017) were strictly adhered during performing all the experiments of the study.

2.3. Estrus cycle measurement

Estrus cycle/heat period is one of the reproductive endpoints to predict the hormonal disturbances. Estrus cycle was determined in ZES exposed F1 rats and controls and the time taken for the completion of estrus was determined. Briefly, three progressions of estrus cycles were determined. Estrus cycle comprises of four phases viz., proestrus, estrus, metaestrus and diestrus and each of which can be determined through vaginal cytology [9,10]. Everyday morning between 6.00 AM to 7.00 am, vaginal smears were collected from the control and experimental rats and carefully visualized under BX 43-phase contrast microscope (Olympus, Japan) for the identification of specific stages (proestrus: epithelial cells; estrus: only cornified cells; metaestrus: leucocytes and cornified cells; diestrus: leukocytes) of estrus cycle[11]. After the determination of estrus cycle, the rats were subjected to fertility efficacy assays.

2.4. Fertility studies

The female rats at the stage of proestrus were cohabited with male rats (100 days old) in a 1:1 ratio. The next day morning, the vaginal fluid collected from the females with the help of Pasteur pipette using physiological saline were carefully visualized and recorded the presence of active sperm under microscope. The existence of active sperm in the vaginal smear was taken into account as day 1

of pregnancy/gestation. The pregnant rats from controls and experimental groups were maintained separately and on gestation day 8 and 18, the rats were subjected to autopsy to validate the loss of pre- and post-implantations per rat and photographed. Further, number of resorptions per rat were also determined at day 18 of autopsy.

The following were determined during the fertility assays using the formulas as mentioned below:

Mating Index (%) = (No. of sperm positive females/No. of pairing) X 100

Fertility Index (%) = (No. of pregnant females/No. of sperm positive females) X 100

Pre-implantation loss (%) = ([No. of corpora lutea – No. of implantations]/No. of corpora lutea) X 100

Post-implantation loss (%) = ([No. of implantations – No. of live fetuses]/ No. of implantations) X 100

Resorptions (%) = (Total no. of resorption sites/ Total no. of corpora lutea) X 100

Resorption index (%) = (Total no. of resorption sites/ Total no. of implantation sites) X 100

2.5. Statistical analysis

The obtained data were processed statistically for six individuals in each group and articulated as mean ± SD. Statistical analysis is determined by using two-tailed ANOVA followed by Bonferroni analysis as post-hoc test (GraphPad Prism, ver. 5.0.3.477) and considered statistical significance at $p<0.05$.

3. Results

3.1. General toxicity

No significant clinical signs of toxicity such as urination, lethargic movements, salivation, vocal sounds, hair loss were observed either in time-mated pregnant rats or in F1 generation rats during their postnatal period. However, in ZES exposed rats at 100 mg/Kg body weight exhibited aggressive behavior. No rats (dams and/or F1 generation female rats) were excluded from the experiment. The developmental landmarks in F1 generation male and female pups of this study were reported by our group(8).

3.2. Mensuration of estrus cycle

The average duration of the estrus cycle in F1 control offspring is 5.16 ± 0.166 days compared to 6.166 ± 0.226, 6.833 ± 0.307, and 8.166 ± 0.307 days in ZES exposed F1 rats at 25, 50 and 100 mg/Kg body weight, respectively.Statistical analysis revealed that significant increase in the mean estrus cycle duration ($F=27.43$; $p<0.0001$) in experimental females when compared to the controls in a dose dependent manner Table **1**.

Table 1: Effect of prenatal exposure to zestoretic on estrus cycle length of female rats at their adulthood.

S. No.	Group	Estrus cycle length
1	Control	5.16 ± 0.166
2	Zestoretic 25 mg/kg BW	6.166 ± 0.223 (19.49)
3	Zestoretic 50 mg/kg BW	6.833 ± 0.307 (32.42)
4	Zestoretic 100 mg/kg BW	8.166 ± 0.307 (58.25)
	F value	F=27.43 *P*<0.0001

Values are mean ± SEM of six individuals. Values are cumulative of three successive cycles.BW: body weight. Values in the parentheses are percent change from control. *P* value at <0.05 was considered as significant.

3.3. Body weight F1 dams

The body weights in ZES exposed and control F1 female rats at days, 8 and 18 were monitored and recorded Table **2**. The findings indicated that there was a significant decrease (*P*<0.0001) in the body weights of ZES exposed dams as compared to controls at 8^{th} (F=57.98) and 18^{th} (F=182.5) day.

Table 2: Effect of prenatal exposure to zestoretic on body weights of female rats at their adulthood.

Body weight (g)	Control	Zestoretic25 mg/kg BW	Zestoretic 50 mg/kg BW	Zestoretic100 mg/kg BW	F- value
On 8^{th} day	206.85±0.15	205.67±0.26 (-0.57)	204.73±0.29 (-1.02)	202.58±0.29 (-2.06)	F=57.98 *P* <0.0001
On18^{th} day	236.28±0.20	233.14±0.27 (-1.33)	231.40±0.48 (-2.06)	227.87±0.26 (-3.56)	F=182.5 *P*=<0.0001

Values are mean ± SEM of three individuals. BW: body weight. Values in the parentheses are percent change from control. *P* value at <0.05 was considered as significant.

3.4. Fertility efficacy of F1 female rats

Table **3** shows the fertility efficacy of ZES exposed rats with various parameters analyzed. ZES at doses 50 and 100 mg/Kg body weight significantly increased (F=75; *P*<0.0001) the mean conception time of F1 females cohabited with control male rats over their respective controls. Significant reduction in the mating index and fertility index was observed in F1 females exposed to ZES at 100 mg/Kg Body weight over controls. Interestingly, the pre- (11% in 25 mg exposed, 21% in 50 mg exposed and 22% in 100 mg exposed) and post-implantation (7% in 25 mg exposed, 10% in 50 mg exposed and 17% in 100 mg exposed) loss in ZES exposed rats was gradually increased with regards to ZES doses over controls at respective time points i.e. on 8^{th} and 18^{th} day at autopsy. The mean number of resorptions per rat in ZES exposed rats (0.66 ± 0.82 in 25 mg exposed, 1.16 ± 0.983 in 50 mg exposed and 1.5 ± 0.5 in 100 mg exposed) was recorded and within the exposed rats, a significant increase was observed in F1 females exposed to higher dose of ZES (p<0.05). The resorption index observed in ZES exposed groups were in the order of 100 mg exposed (17 %) > 50 mg exposed (12 %) > 25 mg exposed (6%). The pre- and post-implantation loss in experimental and controls were show in Figures **2** and **3** respectively.

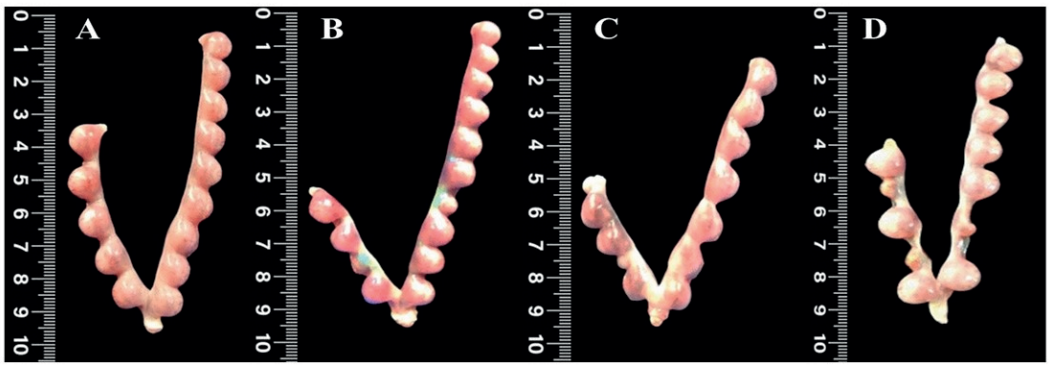

Figure 2: Uterus of rat showing implantations on 8th day of pregnancy. Untreated rats cohabited with normal males (A), whereas Zestoretic exposed rats cohabited with normal males at 25 (B), 50 (C) and 100 (D) mg/Kg body weight. (n= 4/group).

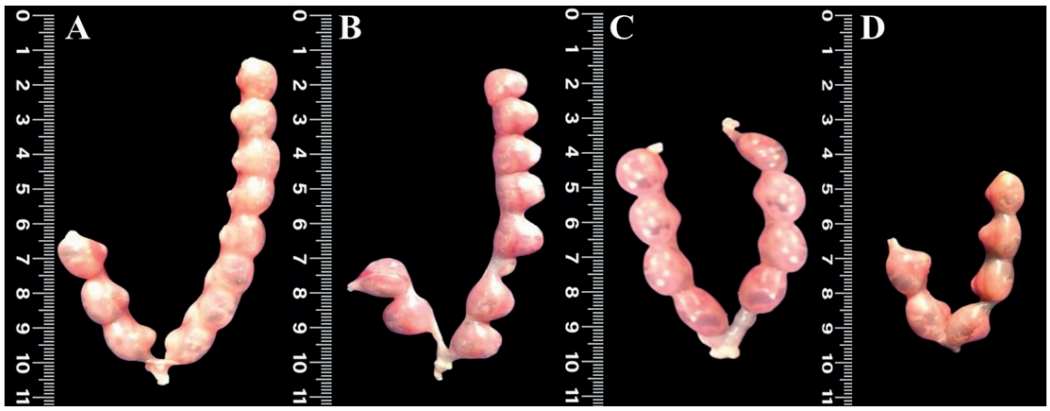

Figure 3: Uterus of rat showing implantations on 18th day of pregnancy. Untreated rats cohabited with normal males (A), whereas Zestoretic exposed rats cohabited with normal males at 25 (B), 50 (C) and 100 (D) mg/Kg body weight. (n= 4/group).

Table 3: Effect of prenatal exposure to zestoretic on reproductive performance in female rats at their adulthood.

Parameters	Control	Zestoretic 25mg/Kg BW	Zestoretic 50mg/Kg BW	Zestoretic 100mg/Kg BW	F-value
Conception time	1.16±0.167	1.66±0.21 (43.10)	3.16±0.167 (172.41)	4.66±0.21 (301.72)	P<0.0001 F=75
Mating index (%)	100(6/6)	100(6/6)	100(6/6)	83(5/6)	
Fertility index (%)	100%	100%	100%	83.33%	
No. of corpora lutea/Rat#	13.33± 0.21	12.66 ± 0.21 (-5.03)	12.33 ± 0.21 (-7.50)	11.66 ± 0.21 (-12.53)	*P*=0.0002 F=13
Implantation %	100	94.73	90.54	87.14	
No. of implantations/ Rat	13.33±0.210	11.33± 0.33 (-11.69)	9.66± 0.33 (-37.64)	9± 0.25 (-55.88)	*P*<0.0001 F=41.22
Pre-implantation loss# (%)	3.74	10.52	21.61	22.85	
No. of live foetus/Rat#	12.33± 0.210	10.50± 0.223 (-14.84)	8.66± 0.421 (-29.76)	7.33± 0.21 (-40.55)	*P*<0.0001 F=62.91
Resorption % #	0	5.26	9.46	12.85	
No. of resorptions/ Rat#	0	0.66±0.816	1.16±0.983	1.5±0.547	*P*=0.0144 F=4.899
Resorptions index (%)	0	6.08	12.10	16.94	
Post-implantation loss# (%)	0	7.35	10.34	17.51	

Values are mean± SEM of six individuals; # n = 3. Values in the parentheses are percent changes from that of control. Significance was considered at *P*< 0.05.

4. Discussion

Hypersensitivity is one of the major reasons for cardiac related problems. Proper medications using anti-hypersensitive drugs are currently available to manage hypersensitivity. However, the side effects of these drugs are not validated properly. Further, as these drugs are not withdrawn suddenly, the establishment of toxicity profile of anti-hypersensitive drugs is of prime concern. In our previous study, we have shown that fetal exposure to ZES at selected doses exhibited developmental toxicity in female pups including delayed vaginal opening. The present findings demonstrated that prenatal exposure to ZES at least in part perturbs female reproductive wellbeing in rats at their adulthood as made known by improper estrus cycle and most strikingly deterioration of fertility efficacy. The present results provide a link between the usage of anti-hypersensitive drugs during pregnancy and the suppressed female reproductive development and performance in rats at their adulthood.

It is well established that the proper menstrual cycle is an inimitable physiological reproductive episode in female mammals which permits potential pregnancy. So far, the exact link between the hypertension and improper menstrual cycle and fertility outcome has not been established[12]. In this study, ZES exposed females exhibited abnormal estrus cycle associated with deteriorated fertility efficacy in rats at their adulthood suggesting changes in the hormones. It is well established that the human ovary comprised of almost all components of renin angiotensin system (RAS) and as such, hormones that mediate RAS could influence the ovarian development[6]. Thyroid hormones (THs) show a key task in the regulation of menstrual regularity, fertility efficacy and proper pregnancy outcomes [13]. Especially during the embryonic period, THs influence maturation of the RAS and functions of cardiovascular and renal system is well acknowledged[13]. Considering the facts that a) maternal THs are key for the development of vital organs including ovarian development, b) ovaries express RAS during fetal period and c) This mediate their actions via RAS, we speculate that the prenatal exposure to ZES may interfere with the RAS of developing ovaries and even its effects persist in rats at their adulthood. It has been shown that LIS treatment at 10 mg/day over a period of four weeks caused a statistically significant decrease in serum free testosterone (T) levels in hypertensive women with polycystic ovarian syndrome, suggesting a link between free T levels and ovarian RAS[14]. Studies of[15], demonstrated that the changes in ovulation, folliculogenesis and prolonged estrus cycle with longevity in rats exposed to LIS, an ACE inhibitor. Interestingly, no abnormal histological observations of ovaries were found in rats treated with LIS. Recent findings of[16] demonstrated that the treatment of *Rhesus Macaque* endocervical cells with amiloride, a diuretic inhibited epithelial sodium channels (ENaC) thereby reduced viscosity of mucus by endocervical cells using particle-based tracking micro rheology technique. This finding also throws a light on the possible link between the cycle reliant on changes of cervical mucus together with a known ion channel cystic fibrosis transmembrane conductance regulator[16].

The F1 female rats belongs to control and experimental groups were able to cohabited with unexposed male rats in a 1:1 ratio and the selected reproductive endpoints like conception time, fertility index, mating index, implantation, live fetuses, number of corpora lutea, resorptions, pre- and post-implantation loss was evaluated. The findings of this study indicated the fertility efficiency of ZES exposed female rats is lower

when compared to controls, which suggesting the embryonic exposure to ZES is effective in reducing female fertility significantly. The findings, increased conception time may reflect altered sexual behavior. Whether, altered sexual desire could be responsible for reduced fertility in ZES exposed female rats, which need to be authenticated further. RAS system in ovaries plays a key role in ovarian steroidogenesis, corpus luteum formation and also oocyte maturation in mammals. The reduced number of corpora lutea in ZES exposed females at 50 or 100 mg/Kg body weight during their fetal period could be due to disturbances at the level of RAS system. The observation that the increased pre- and post-implantation loss in female rats exposed to ZES prenatally at all selected doses could reflect germinal-developmental and embryo-fetal developmental stage disturbances. Studies of[17] announced that the administration of ACE inhibitor, enalapril during pregnancy led to fetal toxicity. Studies of[18] reported that the administration of ACE inhibitors and angiotensin receptor blocker second and third trimesters may exert harmful effects on fetus. The end result of the current study adds to the current understanding on the usage of anti-hypersensitive drugs during pregnancy especially with regards to the usage of ZES. Hence, medical practitioners should be cautious in prescribing these drugs.

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Author Contributions

“Conceptualization, P.N and P.R.R.; methodology, P.N and SBS.; software, P.N.; validation, A.M, S.B.S and P.R.R.; formal analysis, P.N. and R.K; investigation, P.N.; resources, P.R.R and S.A.R.; data curation, G.M and S.A.R; writing original draft preparation, P.N and R.K.; writing review and editing, P.R.R, and S.B.S; visualization, A.M., and G.M..; supervision, P.R.R.; project administration, P.R.R.; funding acquisition, P.N. and P.R.R.;

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Research content

The research content of manuscript is original and has not been published elsewhere.

Ethical approval

The guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision on Animal Experiments, Government of India) and in compliance with Institutional Animal Ethical Committee at Yogi Vemana University, Kadapa, India (Resolution No: YVU/IEAC/PRR/13/2017) were strictly adhered during performing all the experiments of the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

The data presented in this study are available on request from the corresponding author.

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